Prolactin-like Biological Activity in the Pituitary Glands of the Marsupial *Monodelphis domestica* and of the Amphibian *Rana pipiens* Detected by a Colorimetric Nb2 Lymphoma Cell Proliferation Assay (44081)

BÁLINT KACSÓH[1]

Division of Basic Medical Sciences and Department of Pediatrics, Mercer University School of Medicine, Macon, Georgia 31207

Abstract. **An inexpensive and reliable colorimetric microplate version of the Nb2 lymphoma cell proliferation bioassay for prolactin (PRL) was developed and optimized. The useful range of the assay is between 0.1 and 12.8 ng/ml in terms of rat pituitary PRL. The assay can accommodate up to 20 µl sample/well. The physiological relevance of the assay was verified by measuring thyrotropin-releasing hormone (TRH)–induced secretion of PRL in pituitary cultures and in serum samples of neonatal rats. Through the use of the colorimetric Nb2 assay, PRL-like bioactivities were demonstrated in pituitary extracts of the marsupial, *Monodelphis domestica* (1.47 ng PRL/µg protein) and of the amphibian, *Rana pipiens* (1.86 ng PRL/µg protein). Marsupial and amphibian PRLs are predicted to have low specific activities in the Nb2 assay. Since the PRL values were calculated in terms of a rat PRL standard, they probably underestimate the amounts of PRL present. Parallel dose-response curves were obtained with these pituitary extracts and standard rat PRL over a wide range of dilutions. The Nb2 bioassay may serve as a tool for the purification of PRL from these species. The colorimetric version of the Nb2 bioassay may be a useful alternative to traditional Nb2 assays that rely on direct cell count or [^{3}H]thymidine uptake.** [P.S.E.B.M. 1997, Vol 214]

Lactogenic hormones, several cytokines, erythropoietin, and several growth factors belong to the family of the helix-bundle peptides, which share a similar 3-dimensional structure in spite of their diverse primary structures (1). The family of lactogenic hormones includes prolactin (PRL), growth hormone (GH), placental lactogens (PLs), several PRL-like proteins expressed by the placenta, and somatolactin, which has only been described in fish. These hormones are evolutionarily related and display considerable homology in their amino acid sequences, complementary DNA sequences and genomic organization (2, 3). The main source of circulating PRL is the mammotrope cell of the pituitary gland. PRL was named after its role in the regulation of milk secretion. However, it has such a variety of biological actions that it was once proposed that it be renamed "versatilin" (4). Although the mammary gland and milk-production is specific for mammals, PRL is present in fish, amphibians, reptiles, and birds (2, 3, 5). Specific radioimmunoassays (RIAs) have been developed for PRL in several species. These assays are usually highly species specific, and the antibodies may show minimal cross-reactivity with PRL of other species, often making heterologous RIAs either unfeasible or unreliable. Therefore, in the absence of a suitable RIA, monitoring PRL during its chemical isolation may require a bioassay.

[1] To whom requests for reprints should be addressed at Division of Basic Medical Sciences and Department of Pediatrics, Mercer University School of Medicine, Macon, GA 31207.

This work was supported by National Institutes of Health Grant HD31662.

Received June 12, 1996. [P.S.E.B.M. 1997, Vol 214]
Accepted September 10, 1996.

0037-9727/97/2142-0146$10.50/0

Marsupials are excellent models for developmental studies because they give birth to their young at a stage of development that is in several aspects comparable either to the embryonic or the fetal stage of intrauterine development in eutherian mammals. This laboratory has an interest in investigating PRL in the short-tailed gray opossum (*Monodelphis domestica*). *M. domestica* is the only commercially available small marsupial species that is suitable for use as a laboratory animal. *M. domestica* has become a popular model in marsupial and developmental research (6, 7). Unfortunately, no species-specific reagents are available for measuring PRL in *M. domestica*. Although PRL plays a central role in the reproductive function of both marsupial and eutherian mammals, no marsupial PRL has ever been cloned or sequenced, no direct measurements of PRL by RIA have been reported for *M. domestica,* and minimal data are available in any of the marsupial species. The only marsupial PRL for which a partial sequence is available is the PRL of Bennett's wallaby (8). Homologous RIAs have been developed for the Bennett's wallaby and the Eastern grey kangaroo; PRLs of other marsupial species display reduced cross-reactivity in these assays (8, 9). The much needed purification of PRL from *M. domestica* requires the detection of the hormone. In this study, whether PRL-like biological activity can be measured in pituitary extracts of *M. domestica* using an Nb2 cell proliferation assay was determined. In addition, pituitary extracts of an amphibian species (*Rana pipiens*) were also evaluated.

PRL has two distinct types of action, a proliferative and a differentiation-promoting action. Bioassays for PRL have been developed that rely on either or both of these actions. The classical bioassay for PRL is the pigeon crop-sac assay, which is very specific but is relatively insensitive, very labor-intensive, and has poor precision (10, 11). The most sensitive biological assay for PRL is the Nb2 lymphoma cell proliferation assay. The original version of the Nb2 assay for measuring PRL-like biological activity relied on direct cell counts (12). This is relatively labor-intensive, slow, and has a low sample-throughput. Another version of the assay that utilizes the incorporation of [^{3}H]thymidine as a measure of cell proliferation rate has an excellent sample-throughput and precision, but it is expensive and requires special equipment for blotting the cells and measuring radioactivity (13–15). Conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an intensively colored formazan by vital mitochondria has been utilized in cell proliferation and cytotoxicity assays (16). In this paper, a simple, inexpensive and reliable Nb2 assay is described in which PRL-like biological activity is quantified by measuring conversion of MTT.

Materials and Methods

Animals. The animal protocols were approved by the Institutional Animal Care and Use Committee.

Adult female Sprague-Dawley rats were purchased from Harlan Laboratories, Inc. (Indianapolis, IN) and maintained on a 12:12-hr light:dark schedule (lights on at 0600 hr) in a temperature controlled vivarium (20°–22°C, relative humidity 40%–60%). The animals were bred, housed individually from about Day 19 of gestation, and checked for parturition twice a day. The day of parturition was designated Day 0 of postnatal life for the pups. A standard rodent diet and tap water were available to the mothers *ad libitum*. Two-day-old pups from litters with 8–16 pups were used in the experiments.

Short-tailed gray opossums were purchased from the Southwest Foundation for Biomedical Research (San Antonio, TX). Northern frogs were purchased from Hazen Frog Farms (Albany, VT). The animals were used immediately upon receipt.

Generation of Samples for Assay Verification. In vitro *samples.* Rat pituitary hormone response to thyrotropin-releasing hormone (TRH; Peninsula Laboratories, Belmont, CA) was tested *in vitro* using methods described earlier (17) with modifications. Two-day-old rat pups were removed from their mothers and decapitated, and pituitary glands were collected in culture medium. The culture medium was composed of Dulbecco's modified Eagle's medium (DMEM, low-glucose formulation with sodium pyruvate and L-glutamine, Life Technologies, Gaithersburg, MD), pH 7.4, supplemented with 10 m*M* HEPES (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 20 μg/ml L-ascorbic acid, and 0.1% of RIA-grade bovine serum albumin (BSA; Sigma). Pituitaries were placed individually in the wells of a 24-well culture dish (Corning, New York, NY). To stabilize basal secretion, each gland was incubated in 1 ml medium for 3 hr at 37°C in a humidified 5% CO_2/95% air incubator. After the stabilization period, each well was rinsed twice with 1 ml DMEM, and the glands were further incubated in 1 ml/well DMEM for 1 hr to determine the basal secretion rate for each individual gland. The conditioned medium was collected, the wells were rinsed twice with 1 ml DMEM, and the glands were exposed to either DMEM (control) or 10 n*M* TRH. The glands were again incubated for 1 hr, and the conditioned medium was collected from each well. DMEM, 10 n*M* TRH (test substances), and the samples were stored at −20°C until assayed for PRL.

In vivo *samples.* To test the effect of TRH on the circulating levels of PRL in 2-day-old rats, the pups were separated from their mothers for 6 hr. During separation, the pups were kept in a cage filled with wood chips and were covered with a paper towel to reduce heat loss. The pups were not fed during separation. The pups were numbered and weighed (precision: 0.1 g) at the time of separation. Each litter was divided into control and treatment groups to assure the use of littermate controls. An even distribution of males and females among the treatment groups was attempted in each litter. TRH (10 ng/g body wt) was injected intraperitoneally in physiological saline solution at a volume dose of 1 μl/g body wt. Each pup was decapitated 15

min later, and trunk blood was collected. After clotting overnight at 4°C, serum was collected and stored at −20°C until assayed for PRL.

Extraction of Pituitaries. Frogs and adult female opossums were decapitated, their pituitary glands were removed and stored frozen until extraction by the method described by Ishikawa *et al.* (18). In brief, the pituitary glands were homogenized in ice-cold 0.1 *M* phosphate-buffered saline (PBS, pH 7.0) containing 1 *M* urea. The homogenate was frozen and thawed three times and microfuged, and the supernatant was dialyzed (MW cutoff: 3500) against 0.1 *M* ammonium bicarbonate at 4°C. The dialyzed extract was aliquoted and lyophilized. The lyophilized material was dissolved in 10 m*M* sodium bicarbonate, and serially diluted either in phosphate-buffered saline (PBS, pH 7.4) for measurement of total protein concentration, or in FM-2 (the assay medium used in the Nb2 lymphoma test) for measurement of PRL-like biological activity.

The protein concentration of the dialyzed pituitary extract was estimated in a microplate Bradford assay (Bio-Rad, Richmond, CA) using BSA as a standard. PRL-like bioactivity was estimated by comparing the cell-proliferation induced by the opossum and frog pituitary extracts with that induced by the purified rat PRL standard (B-6; National Hormone and Pituitary Program [NHPP] and National Institute of Diabetes, Digestive, and Kidney Diseases [NIDDK], Bethesda, MD).

Nb2 Lymphoma Proliferation Bioassay for Prolactin. *Maintenance of the Nb2 cell line.* The maintenance medium (termed FM-1) for the Nb2 cells was Fisher's medium (Life Technologies), pH 7.6, supplemented with 10% defined fetal bovine serum (FBS), 10% defined horse serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 0.1 m*M* 2-mercaptoethanol (Sigma). Cells were propagated by seeding 20 ml FM-1 with Nb2 cells at a density of 15,000 cells/ml in T-75 tissue culture flasks (Corning), and culturing the cells in a humidified 5% CO_2/95% air atmosphere at 37°C. The cells grew in a suspension culture; logarithmic growth occurred during the first 72 hr after seeding (Fig. 1). The doubling time of the cells was 10.75 hr, considerably shorter than the 20 hr reported by Tanaka *et al.* (12). After 72 hr in culture, the cells were pelleted in a 50-ml sterile polypropylene centrifuge tube at room temperature (20°–22°C) at 800 rpm for 10 min. The used culture medium was aspirated and discarded; the cells were resuspended in 10 ml FM-2 (see below). Cell counts were obtained with a hemacytometer, and a new flask was seeded with Nb2 cells as described above. The surplus cells were either used for a PRL bioassay or discarded. Using this protocol, Nb2 cells have been maintained over 250 passages without any detectable change in their responsiveness to PRL.

Optimization of the colorimetric Nb2 cell proliferation assay. Due to the inherent lactogenic activity of FBS, an FBS-free assay medium was needed for measurement of PRL in biological samples. This medium (termed FM-2) was identical with FM-1 except that FBS was omitted. It is important to note that several batches of horse serum need to be tested because they differ in their endogenous PRL concentrations and some batches may be toxic for Nb2 cells. This observation is in agreement with earlier reports (19). Once a batch is identified that maintains the Nb2 cells but does not significantly promote their rate of proliferation, a larger stock needs to be ordered and stored at −20°C.

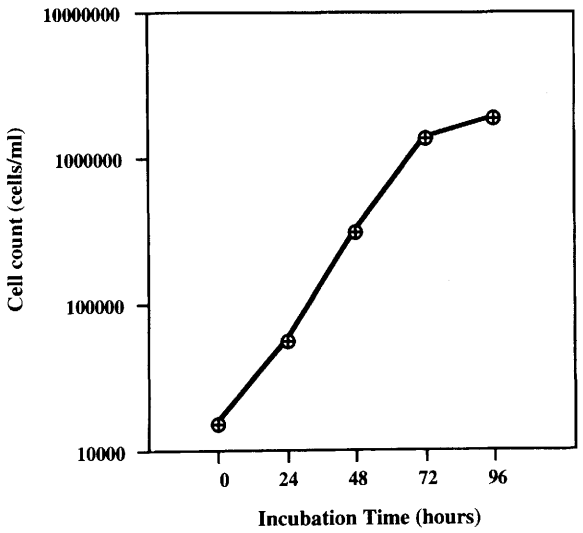

Figure 1. Time-dependent proliferation of Nb2 cells in suspension cultures maintained in FM-1 medium. The cell count used for seeding was 15,000 cells/ml. The ordinate displays mean cell counts and standard deviations. During the first 72 hr after seeding, the relationship between time and cell count was described by the equation lg(count) = 4.141 + 0.028*t*, where *t* is the time in hours ($r = 0.999$, $P = 0.0013$). The doubling time of the cells was 10.75 hr.

In order to render the Nb2 cells quiescent, 5 ml of the 10-ml suspension (the pelleted cells that were resuspended in FM-2, see above) was added to 15 ml of FM-2 in a T-75 flask. After 24 hr of maintenance in a humidified 5% CO_2/95% air atmosphere at 37°C, the cells were pelleted again and resuspended in 10 ml FM-2, and cell counts were obtained. The total cell count after this 24-hr incubation exceeded the initial cell count by less than 3-fold.

In preliminary experiments (not shown), various cell numbers were tested for seeding 96-well flat-bottomed tissue culture plates (Corning). A seeding density of 5000 cells/well in 80 μl/well FM-2 (i.e., 62,500 cells/ml) was selected. This cell suspension was prepared, poured into a disposable sterile polyethylene reagent reservoir (Costar, Cambridge, MA), and dispensed into the microplate with a 12-channel adjustable pipette (12-pette, Costar) in a sterile laminar flow hood. After seeding the plate with cells, a 20-μl volume (representing the standard or the sample) was added to each well, bringing the final volume to 100 μl/well and the cell density to 50,000 cells/ml. Rapid and precise pipetting of samples and standards was crucial. To meet these requirements, all calibration points and samples were first dispensed into autoclaved 1-ml capacity microtiter tubes (Bio-Rad) racked in a microplate format, and the 20

μl/well volumes were then transferred with a 12-pette. The typical arrangement in a microplate is shown in Figure 7 below. Each set of the quadruplicates was transferred from a row of racked microtiter tubes; pipetting took less than 90 sec/plate. The cells were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for various times as indicated in the figures.

Standard PRL (B-6; NHPP, NIDDK) was dissolved in 10 m*M* sodium bicarbonate (1 μg/μl), diluted in FM-2 to a concentration of 5.325 μg/ml, and stored at −70°C in aliquots of 200 μl/tube. For the assay, an aliquot was diluted to a final volume of 2.6 ml in FM-2 (409.6 ng/ml), then serially diluted in FM-2 with a dilution factor of 2, and pipetted into microtiter tubes (see above).

PRL-induced cell proliferation was assessed by conversion of MTT (Sigma) into formazan. MTT was dissolved in 25 m*M* PBS (pH 7.6) at the concentration of 5.5 mg/ml, sterile-filtered, and stored refrigerated in the dark for up to 12 months. After the Nb2 cells were incubated with standard PRL for either 24, 48, 72, or 96 hr, 10 μl MTT solution was added to each well with an Eppendorf repeater pipet using a 0.5-ml capacity combitip (Brinkmann Instruments, Westbury, NY) and a 200-μl pipet tip fitted onto the combitip. This pipetting step took approximately 90 sec/plate. The plates were then incubated for either 2 or 4 hr in the CO_2 incubator.

Color development was stopped and the formazan product was solubilized by adding 100 μl/well of solubilizing agent with a 12-pette. The solubilizing agent consisted of 0.1 *M* acetate buffer (pH 4.7) containing 20% (w/v) sodium lauryl sulfate (SDS) and 50% (v/v) N,N-dimethylformamide (DMF) (16). The order of mixing the reagents was important in the preparation of the solubilizing agent: DMF and water were mixed, and after SDS was dissolved completely (a warm water bath was needed), acetic acid (at a concentration of 40 m*M*) and sodium acetate (at a concentration of 60 m*M*) were added. Finally, the pH was adjusted to 4.7 with 5 *N* hydrochloric acid.

After the addition of the solubilizing agent, the plates were wrapped in plastic foil and incubated in the dark at room temperature (20°–22°C) for 2 days. Absorbances were determined with an automatic ELISA plate reader at the wavelength of 595 nm.

Measurement of PRL in samples. Standard PRL and pituitary extracts were serially diluted in FM-2 (dilution factor of 2). Rat serum samples were diluted 1:4. Conditioned DMEM samples were used undiluted in the assay. Twenty microliters of the standard or the samples were added to each well. Standards and samples were analyzed in quadruplicate. A full assay calibration curve and up to 10 samples were included in each plate. The cells were incubated for 48 hr at 37°C in a humidified 5% CO_2/95% air incubator. After adding MTT to the wells, the cells were incubated for another 2 hr, and the reaction was terminated. All samples from each experiment were measured in the same assay to avoid interassay variability.

Statistical Analysis. The Nb2 and Bradford assays were analyzed with the AssayZap program (Biosoft, Cambridge, United Kingdom). Statistical evaluation of the data was performed using the StatView program (Abacus Concepts, Inc., Berkeley, CA) and a Power Macintosh 6100/66 personal computer (Apple Computer Inc., Cupertino, CA). The data are shown as mean + SEM (n), where n is the number of individual pituitary glands or individual pups (i.e., individual hormone measurement data points). The number of litters used in each experiment is given separately. The data were analyzed by unpaired Student's t test. Differences were considered statistically significant whenever P values were 0.05 or less.

Results

The optimal culture time of PRL-induced cell proliferation was evaluated with a fixed 2-hr incubation with MTT. The optimal culture time was found to be 48 hr. A 24-hr culture time resulted in a shallow dose-response curve, whereas a 72-hr culture time led to a hooked calibration curve with the maximal dose of PRL resulting in a submaximal formazan production. This was even more evident after a 96-hr culture time (Fig. 2).

The 24- and 48-hr culture times were further evaluated with 2- and 4-hr incubations with MTT (Fig. 3). The response of formazan production to PRL was characterized by comparing absorbances of the maximally stimulated wells (B_{max}; 20 μl of 409.6 ng/ml rat PRL) and of those receiving FM-2 without PRL (*NSB*), using the formula of (B_{max} − blank)/(*NSB* − blank), where "blank" is defined as the absorbance produced by all components of the assay in the absence of cells. The 24-hr culture time resulted in a shallow dose-response curve independent of the incubation time with MTT; the response ratio was 1.99 in case of the 2-hr incubation and 1.95 in case of the 4-hr incubation with MTT. The 48-hr culture time resulted in a response ratio of 4.38 in case of the 2-hr and 3.42 in case of the 4-hr incubations with MTT. The decline in the response ratio indicated that the linear phase of the formazan production was already exceeded by the 4-hr incubation. In addition, the dose-response curve obtained with 4-hr incubation with MTT became slightly hooked. It was therefore concluded that the optimal assay conditions were a 48-hr culture time followed by a 2-hr incubation with MTT.

Several assays were performed, using these optimized conditions, and the assay characteristics were calculated (Table I). The coefficients of variation (CVs) shown in the table were calculated from PRL concentrations; in terms of optical densities, the CVs averaged 2.62%. Based on 78 assay plates, the quality of fit averaged 0.999897 (range: 0.999733–0.9999733). The useful range of the assay was between 0.1 and 12.8 ng/ml in terms of rat pituitary PRL. The detection limit was 0.05 ng/ml at a (maximal) sample volume of 20 μl/well (i.e., 1 pg/well). A typical calibration curve is shown in Figure 4. The assays included in the calculations were performed over a 6-month period using

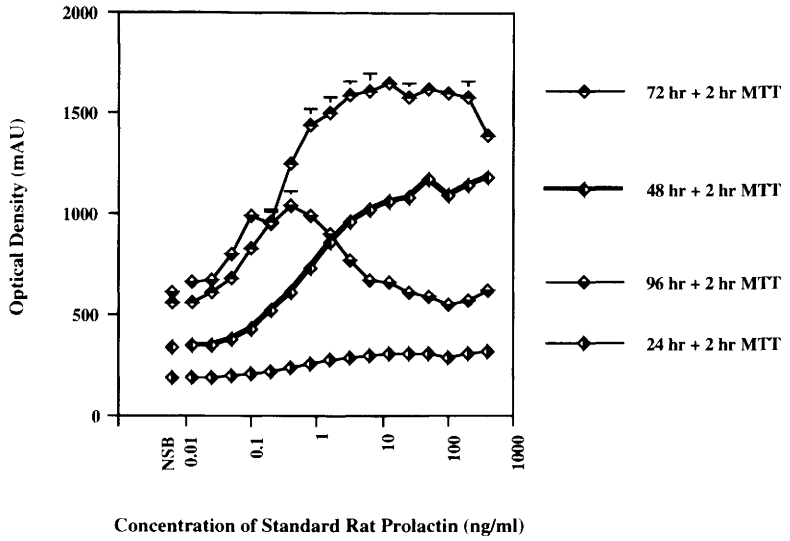

Figure 2. Dose- and time-dependent proliferation of Nb2 cells as detected by MTT conversion in a microplate assay. The initial cell count was 5000 cells/well; 20 µl/well of rat PRL (B-6 from NHPP, NIDDK) was added to each well at the working dilutions indicated in the figure (final volume: 100 µl/well). The optical density obtained in the absence of rat PRL is displayed as NSB; the maximal dose of PRL was 409.6 ng/ml (81.9 ng/ml in terms of final concentration; i.e., 8.19 ng/well). The maximal dose of PRL was equipotent with 10% fetal bovine serum (not shown). Each point is the mean + SD of four measurements. Four identical plates were prepared simultaneously, and were processed for MTT-conversion assay every 24 hr after exposure to PRL.

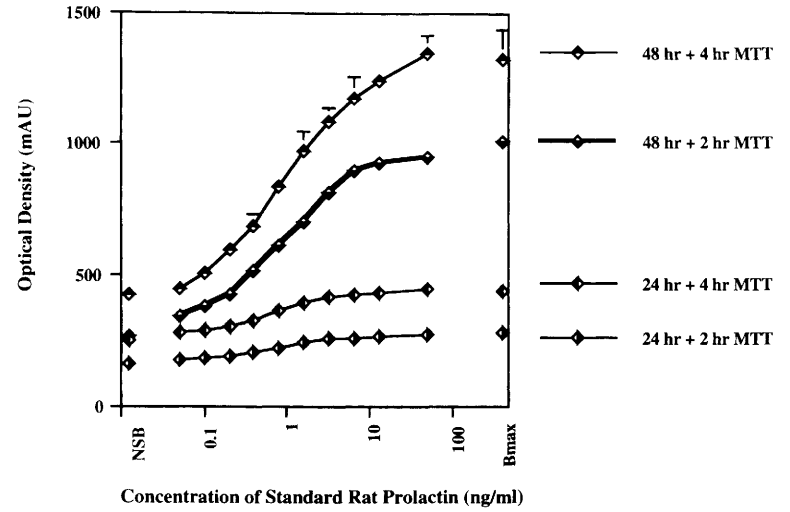

Figure 3. Effect of incubation time with MTT on the dose- and time-dependent proliferation of Nb2 cells in a microplate assay. The initial cell count was 5000 cells/well; 20 µl/well of rat PRL (B-6) was added to each well at the working dilutions indicated in the figure (final volume: 100 µl/well). The optical density obtained in the absence of rat PRL is displayed as NSB; the maximal dose of PRL (B_{max}) was 409.6 ng/ml. Each point is the mean + SD of eight measurements. Four identical plates were prepared simultaneously, and were processed either 24 or 48 hr after exposure to PRL; the cells were exposed to MTT for either 2 or 4 hr.

the same batch of defined horse serum. The detection limit and the 50% effective dose (ED_{50}) of the assay is slightly influenced by the quality of the horse serum (not shown). However, by selecting appropriate batches of horse serum, the assay parameters shown in Table I have been maintained over a period of two and a half years without significant changes.

Circulating levels of PRL are very low in neonatal rats (14, 15). Pituitary glands of neonatal rats secrete minute quantities of PRL also *in vitro* (13, 15). Especially in individual pituitary culture or serum samples of neonatal rats, PRL concentration may be difficult to measure by RIA. To verify the colorimetric Nb2 assay with biological fluid samples, serum samples and culture media conditioned with neonatal rat pituitary glands were used. TRH is a well-established secretagog of PRL (3, 20). Therefore, TRH-induced PRL-responses were evaluated. In pituitary cultures of 2-day-old rats, exposure to 10 n*M* TRH for 1 hr resulted in a significant ($P < 0.0001$, unpaired t test) increase in the secretion of PRL-like biological activity (Fig. 5). Twenty microliters per well of either nonconditioned DMEM or 10 n*M* TRH (in DMEM) did not stimulate proliferation of Nb2 cells (not shown). *In vivo,* TRH (10 ng/g body wt) significantly ($P < 0.0001$, unpaired t test) increased circulating

Table I. Assay Characteristics

Dose of PRL (ng/ml)	Interassay CV (%)	Intraassay-interplate CV (%)	Intraassay-intraplate CV (%)	Accuracy (% recovery of the nominal concentration)	CV (%) of accuracy
ED_{20} = 0.17	18.4	23.1	—	—	—
At 0.20 ng/ml	—	—	16.1	95.0	7.4
ED_{50} = 1.245	12.9	15.0	—	—	—
At 1.60 ng/ml	—	—	13.7	107.9	9.2
ED_{80} = 9.77	18.1	34.5	—	—	—
At 12.8 ng/ml	—	—	34.3	102.2	27.7

Note. The assay parameters were calculated from 10 assays performed over a 6-month period. In each assay 6 to 12 plates were used (a total of 78 plates). The AssayZap software calculates effective doses (EDs) of ED_{20}, ED_{50}, and ED_{80} for each calibration curve (i.e., each plate); interassay and intraassay-interplate coefficients of variation (CVs) were calculated from these values. To assess within-plate precision and accuracy, for each plate CVs and recovery were calculated from the calibration points closest to the ED values calculated by the software. The table shows the average of the intraassay-intraplate CVs; the CVs of accuracy were calculated from the standard deviations belonging to the respective recoveries.

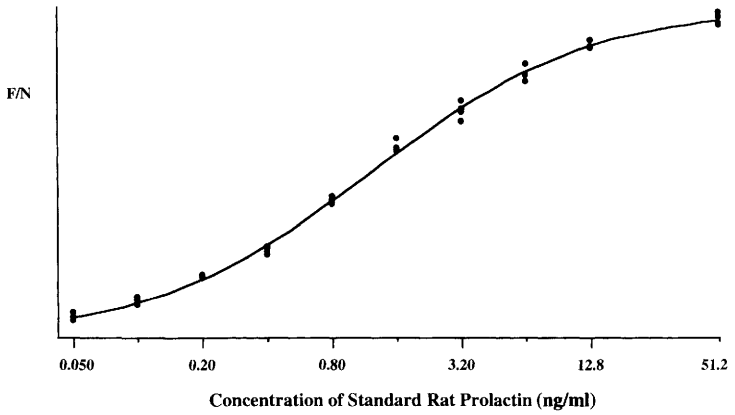

Figure 4. Representative calibration curve of the optimized colorimetric Nb2 assay as displayed by the AssayZap software. The initial cell count was 5000 cells/well; 20 µl/well of rat PRL (B-6) was added to each well at the concentrations (working dilutions) indicated in the figure (final volume: 100 µl/well). Each dot represents a well in the calibration; the curve was calculated from quadruplicate measurements.

concentrations of PRL-like biological activity (Fig. 6). Unstimulated and TRH-stimulated levels of PRL in the samples resulted in robust differences of formazan production that were readily visible with the naked eye (Fig. 7).

After completing the validation of the colorimetric Nb2 assay, it was determined whether pituitary extracts of opossums and frogs contained PRL-like biological activities. Aliquots of lyophilized pituitary extracts (approximately 0.2 gland/aliquot for opossums, 0.4 gland/aliquot for frogs) were dissolved in 50 µl of 10 m*M* sodium bicarbonate. Serial dilutions of BSA and the pituitary extracts in PBS revealed parallel dose-response curves in the Bradford assay (not shown). The protein concentrations were 333 µg/ml for opossum pituitary extracts (approximately 83.25 µg/gland) and 566 µg/ml for frog pituitary extracts (approximately 70.75 µg/gland). Serial dilution of standard rat pituitary PRL and the pituitary extracts in FM-2 demonstrated parallel dose-response curves in the Nb2 assay (Figs. 8 and 9). PRL-like biological activities were calculated at 487.9 ng/ml in opossum pituitary extracts (122 ng/gland) and at 1053.4 ng/ml in frog pituitary extracts (131.7 ng/gland). On a protein basis, PRL-like biological activities in opossum and frog extracts were 1.47 ng/µg and 1.86 ng/µg, respectively.

Discussion

The colorimetric version of the Nb2 assay presented in this paper provides a simple, inexpensive, highly sensitive, and reliable method for measuring PRL-like biological activities in serum, conditioned culture media and pituitary extracts. The assay has an excellent sample-throughput: this laboratory routinely runs assays containing 120 samples. The detection limit of the assay (in terms of final concentration of rat PRL in the Nb2 cell culture) is approximately 10 pg/ml, which is practically identical with that described by Tanaka *et al.* (12) for human PRL using a direct cell count version of the Nb2 assay. The colorimetric Nb2 assay was compared to the [^{3}H]thymidine-uptake version of the assay in a preliminary communication. It was concluded that both versions of the assay measure quantitatively similar PRL values in biological samples with an excellent correlation (21). The precision of the colorimetric assay represents an improvement over an Nb2 assay in which cell

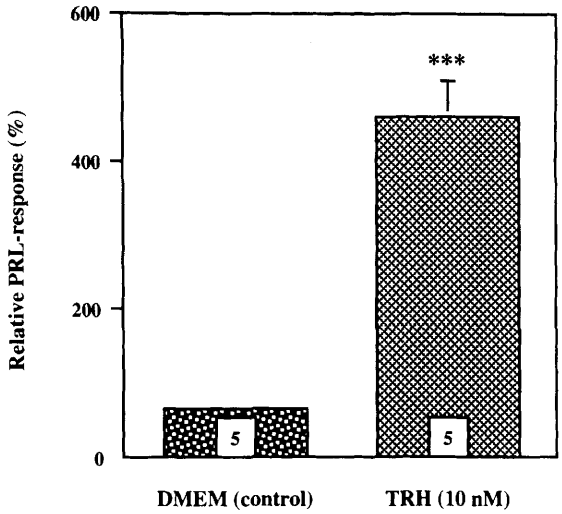

Figure 5. Thyrotropin-releasing hormone- (TRH-) induced PRL secretion of pituitary glands obtained from 2-day-old rats. The number of individual pituitary glands (data points) are shown at the bottom of the columns. The pups used in this study were obtained from three litters. The PRL-response was calculated for each individual gland as the percentage of the PRL concentration in the conditioned culture medium (DMEM) upon an exposure either to DMEM or to 10 n*M* TRH compared with basal secretion (a prior exposure to DMEM). The basal secretion rate was 0.78 ± 0.26 ng/ml/hr/gland (n = 10). TRH significantly stimulated PRL secretion (Student's unpaired t test: $P < 0.0001$).

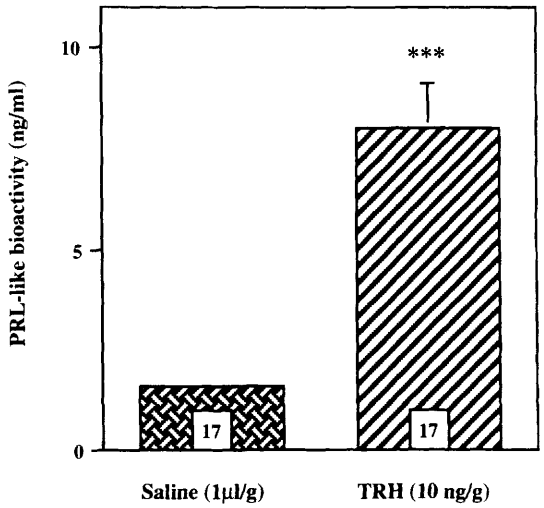

Figure 6. TRH-induced levels of circulating PRL in 2-day-old rats. The number of individual pups (data points) are shown at the bottom of the columns. The pups used in this study were obtained from three litters. The pups were injected intraperitoneally; trunk blood was collected 15 min later. PRL was measured in serum. TRH significantly stimulated PRL secretion (Student's unpaired t test: $P < 0.0001$).

proliferation was assessed by incorporation of [^{3}H]thymidine (14, 15).

While these present experiments were in progress, two groups have reported Nb2 assays based on MTT-formazan conversion (22, 23). It is interesting to note that Ealey *et al.* (23) seeded the wells with 20,000 cells and cultured the cells for the assay for 96 hr. In the Nb2 assay reported in the present study, only 5000 cells/well were seeded, yet maximally stimulated cultures overgrew between 48 and 72 hr.

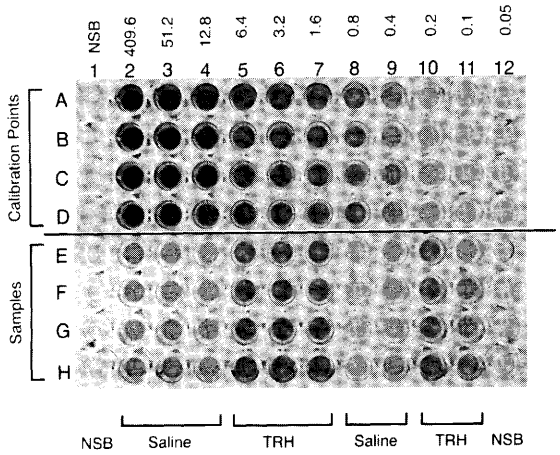

Figure 7. Photograph of an Nb2 assay microplate. The upper half of the plate (Rows A–D) contains the calibration points (standard rat PRL); the lower half of the plate (Rows E–H) contains samples. Concentrations of PRL (ng/ml) are given for the working dilutions. The samples (neonatal sera) were diluted 1:4 in FM-2 assay medium. Standards and samples were measured as 20 µl/well quadruplicates arranged within the columns of the plate. NSB: color development in the absence of PRL. The serum samples were obtained from 2-day-old rats injected with saline (control) or 10 ng/g TRH.

At high doses of PRL, extensive cell death occurred by 96 hr. The unexpected differences between the suitable culture times in these studies might be attributed to the difference in the culture media used (RPMI-1640 versus Fisher's medium). The colorimetric Nb2 assay reported in this paper requires fewer cells/well, shorter microplate culture times and fewer pipetting steps than the assay described by Ealey *et al.* (23). It also avoids pelleting the cells by centrifugation of the microplates and aspiration of the supernatants, steps utilized in the method of Adler *et al.* (22). It is difficult to compare the sensitivity of these assays to the Nb2 assay reported in the present paper. Ealey *et al.* (23) used human GH as a standard, whereas Adler *et al.* (22) used PRLs from several species. However, the only rat PRL Adler *et al.* (22) used was RP-3. This is a standard that is distributed by the NHPP for RIA purposes and contains a cytotoxic preservative, which explains why Adler *et al.* (22) observed diminished cell proliferation at doses exceeding 2 ng/ml. It is also unclear whether the concentrations given in their paper refer to working dilutions or final concentrations.

The Nb2 cell line was isolated from a lymph node of an estrogenized male rat that developed a pre-T-cell lymphoma (24, 25). The Nb2 assay is fairly specific for PRL. Proliferation of the Nb2 cells is dependent upon the presence of lactogenic hormones: these include PRL and PL from all mammalian species studied and human (but not subprimate) GH; the placental PRL-related proteins are ineffective (12, 26). Proliferation of the Nb2 cells is not promoted by classical growth factors (12), but certain interleukins (ILs), such as IL-2 and IL-7 are effective (13, 15, 27). However, pituitary and circulating concentrations of these ILs are low and the Nb2 assay specifically measures lactogenic hormones in these biological samples.

The Nb2 assay, unlike the pigeon crop-sac assay, uti-

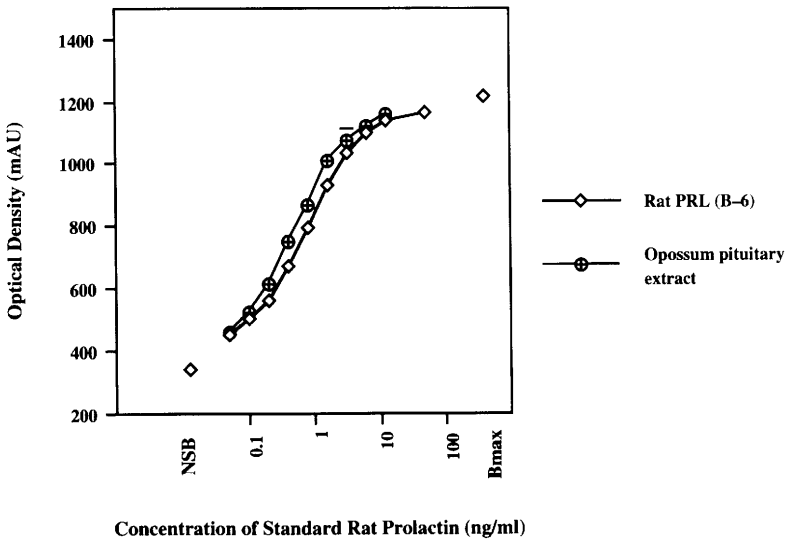

Figure 8. Parallel dose-response curves obtained with rat PRL and opossum pituitary extract. The values are shown as mean + SD; each data point was calculated from quadruplicate measurements. For both preparations the dilution factor was 2. Lyophilized opossum pituitary extract (equivalent of 0.2 gland) was dissolved in 50 µl of 10 n*M* sodium bicarbonate and diluted in Nb2 assay medium (FM-2). The top dose of the opossum pituitary extract was a 25-fold dilution, (i.e., 13.32 µg/ml [266 ng/well] total protein).

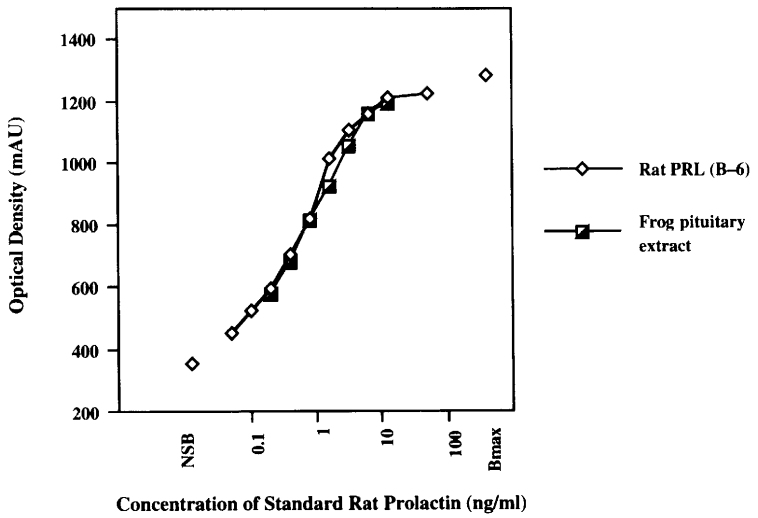

Figure 9. Parallel dose-response curves obtained with rat PRL and frog pituitary extract. The values are shown as mean + SD; each data point was calculated from quadruplicate measurements. For both preparations the dilution factor was 2. Lyophilized frog pituitary extract (equivalent of 0.4 gland) was dissolved in 50 µl of 10 m*M* sodium bicarbonate and diluted in Nb2 assay medium (FM-2). The top dose of the frog pituitary extract was a 100-fold dilution (i.e., 5.66 µg/ml [113 ng/well] total protein).

lizes only the proliferative action of PRL. PRL exerts it effects *via* specific receptors that belong to the cytokine receptor superfamily. PRL receptors have a long and a short form which are generated by alternative splicing of the primary transcript of the same gene (28). The Nb2 cells express a deletion-mutant intermediate-length PRL receptor (29). Although the deletion affects the intracellular domain of the receptor, this does not impair postreceptor signaling (30), and actually results in an increased affinity for PRL (31).

The PRL-like biological activity detected in the opossum and frog pituitary extracts most probably represents PRL and not GH because all non-primate mammalian and avian GHs tested were ineffective in the Nb2 bioassay (12, 32). The quantities of PRL-like biological activities in opossum and frog pituitary extracts were considerably lower than found in rat, ovine, bovine, porcine, or Bennett's wallaby pituitaries as detected by homologous RIAs or pigeon crop-sac bioassay (8, 18, 33). All tetrapod PRLs, but not teleost PRLs, are biologically active either in pigeon crop-sac assay or in mammary milk secretion-promoting assays (2, 5). Therefore, it is not unexpected that opossum and frog PRLs were recognized by the Nb2 type rat PRL receptors.

The low PRL-like activities found in opossum and frog pituitary extracts are most likely related to two factors: the assay system and the evolutionary relationship of PRL in these species with those of the primate, ruminants, or rodents. When compared with bullfrog PRL, mammalian PRLs can be classified into three sequence homology

groups: the first group consists of the horse, pig, and fin whale PRL with 68% identity; the second group, human bovine, ovine, and elephant PRL with 60% identity; and the third group, rodent PRL with 50% identity (5). According to the partial sequences available for kangaroo PRL and GH, these hormones display the highest homology with their porcine and equine counterparts, respectively (8). Therefore, it can be speculated that similar to equine and porcine PRL, marsupial and amphibian PRL would have a low specific activity in the Nb2 bioassay, and that the hormone values calculated for opossum and frog pituitary extracts using a rat PRL standard underestimate the pituitary PRL content in these species. This conclusion is also supported by the finding that kangaroo PRL has low specific activity in pigeon crop-sac bioassay (9).

The low potency of equine PRL in the Nb2 assay system is actually exploited by the use of horse serum. Porcine PRL also has low specific activity in the Nb2 assay: its ED_{50} value is about 16- to 20-fold higher than the ED_{50} values calculated for rat or ovine PRLs (unpublished observation). Others have also demonstrated low biological potency of porcine PRL in Nb2 assays, while in homologous bioassays the same porcine PRLs were highly active (34, 35). The low specific activity of porcine PRL in the Nb2 assay may be in part related to its high proportion of glycosylated PRL; glycosylated PRLs from all species studied are less potent in Nb2 assays than their nonglycosylated counterparts (36, 37). Avian (turkey and chicken) PRLs have been proven to promote Nb2 cell proliferation; their potency was comparable to porcine PRL that was used as a standard in that study (32). Bullfrog PRL has 75% and 72% homology with turtle and chicken PRL, respectively (5). Taken together, these findings support the view that amphibian, reptilian, avian, marsupial and a group of eutherian mammalian PRLs (horse, pig, and fin whale) may have similar structural features resulting in a low potency in the Nb2 bioassay. Glycosylated porcine PRL is more active than nonglycosylated porcine PRL in homologous radioreceptor and bioassays (34). It seems plausible that a high proportion of marsupial PRL is glycosylated and that PRL receptors of marsupials preferentially recognize the glycosylated form of marsupial PRL.

The author expresses his thanks to Dr. A. F. Parlow, the NHPP, and the NIDDK for providing biologically active rat PRL. The author is grateful to Dr. W. C. Hymer of Pennsylvania State University for providing the initial batch of Nb2 cells.

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